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Grant Number DAMD17-94-J-4117

TITLE: Role of Accessory Molecule(s) in Endotoxin-Endothelial Interactions and Endothelial Barrier Dysfunction

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REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

OMB No. 0704-0188

1. AGE Y USE ONLY *(Leave blank)*

2. REPORT DATE
July 1997

3. REPORT TYPE AND DATES COVERED
Annual (1 Jun 96 - 31 May 97)

4. TITLE AND SUBTITLE
Role of Accessory Molecule(s) in Endotoxin- Endothelial Interactions and Endothelial Barrier Dysfunction

5. FUNDING NUMBERS
DAMD17-94-J-4117

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland
School of Medicine
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

This grant has been renegotiated and the new technical objectives are:

1) To determine whether LPS induces tyrosine phosphorylation of endothelial cell proteins that can be coupled to changes in barrier function.

2) To determine whether this LPS-induced endothelial intercellular gap formation and barrier dysfunction is due to zonula adherens and/or focal adhesion disassembly.

3) To determine which protein substrates are the targets of LPS-induced tyrosine phosphorylation.

14. SUBJECT TERMS
endothelium, barrier function, endotoxin, tyrosine phosphorylation, actin cytoskeleton, zonula adherens

15. NUMBER OF PAGES
18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Simeon E. Goldblum 5/21/97

PI - Signature Date

(4) TABLE OF CONTENTS

(5) Introduction:

We have studied the direct impact of endotoxin or bacterial lipopolysaccharide (LPS) on pulmonary vascular endothelial cell (EC) barrier function (1-3). We have found that LPS induces (1) tyrosine phosphorylation of a 65kDa protein, paxillin (3), (2) actin disassembly (2), and (3) opening of the paracellular pathway (2,3). The tyrosine phosphorylation events were clearly prerequisite to the downstream events of actin depolymerization, opening of the paracellular pathway, and loss of endothelial barrier function.

Paxillin is an ~68kDa, highly conserved protein that can be immunolocalized to the specialized cell-matrix adherens junctions, the focal adhesions (FA) (4). That paxillin is present in the FA in relative low abundance suggests more of a regulatory than a structural role. Paxillin has a number of features that favor it as a signaling molecule. Paxillin is phosphorylated on tyrosine residues to a high stoichiometry (20-30% of total cellular pool) in response to numerous agonists (4-8). Interestingly, it is also tyrosine phosphorylated during cellular events associated with remodeling of the actin cytoskeleton (4-8) and in response to certain stimuli, its phosphorylation requires an intact actin cytoskeleton (6-8). Paxillin is equipped with multiple domains predicted to participate in protein-protein interactions including a proline-rich SH3-binding motif that specifically interacts with *Src* family kinases (9-11) five SH2-binding motifs several of which interact with the adaptor molecule, *crk* (9,10) and four LIM domains (9,10), one of which targets paxillin to FAs (12). In addition, paxillin binds to *csk*, FA kinase (FAK), vinculin, talin, and tensin (4). Multiple PTKs have been shown to directly

phosphorylate paxillin on tyrosine including several members of the *Src* family, FAK, and *csk* (4). Interestingly, several established mediators of increased vascular permeability, including bradykinin, platelet-activating factor, α-thrombin, and TNFα (13-16), each have been shown to stimulate tyrosine phosphorylation of paxillin. That paxillin is tyrosine-phosphorylated in response to numerous and diverse stimuli, can serve as a substrate for multiple PTKs, and specifically associates with multiple signaling/adaptor molecules, is compatible with a pivotal role within multiple interacting signaling pathways.

We now report preliminary results of studies on paxillin-protein interactions with an established component of the zonula adherens (ZA), β-catenin. Further, we have studied the impact of the LPS stimulus on the ZA state of assembly including possible proteolysis of both β-catenin and γ-catenin.

(6) BODY

A. Experimental Methods:

LPS Preparation: LPS phenol-extracted from *Escherichia coli* serotype 0111:B4 (Sigma Chemical Co., St. Louis, MO) was suspended in PBS at 5mg/ml and the stock solution was stored at 4°C. For experiments, the LPS stock solution was mixed with supplemented tissue culture media as described below.

EC Culture: Bovine pulmonary artery EC, obtained from the American Type Culture Collection (Rockville, MD), were cultured at 37 °C under 5% CO_2 in Dulbecco's Modified Eagle's Medium (Sigma) enriched with 20% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UT), 5 mM L-glutamine, nonessential amino acids, and vitamins, in the presence of penicillin (50 u/ml) and streptomycin (50 µg/ml) (Sigma). The cells were washed and gently detached with a brief (1-2 min) trypsin (0.5 mg/ml) (Sigma) exposure with gentle agitation followed immediately by trypsin-neutralization with FBS-containing medium. The cells were counted and suspended in medium for immediate seeding of tissue culture dishes or barrier function assay chambers.

Coimmunoprecipitation for Paxillin-Binding Proteins: EC were seeded in 100-mm cell culture dishes (Corning Inc., Corning, NY) at a density of 1.5 x 10^6 cells/dish and cultured for 72h. Monolayers were exposed to LPS (100ng/ml, 1h) or media alone. The cells were then washed x2 with ice-cold PBS containing 1 mM vanadate. The cells were then lysed for 15 min with ice-cold modified RIPA lysis buffer [50 mM Tris-HCl (pH 8.0), 1% NP-40, 0.25% sodium

deoxycholate, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 μg/ml DNAse, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM pyrophosphate, 1 mM phenylarsine oxide (PAO), 500 μM P-nitrophenyl phosphate, 1 mM dithiothreitol (DTT)]. The EC lysates were incubated overnight with anti-paxillin antibody (0.25 μg/ml) (Transduction Laboratories) at 4 °C. The resultant immune complexes were immobilized by incubation with anti-mouse IgG cross-linked to agarose (Sigma) for 2 h at 4 °C. The pellet was isolated following centrifugation, washed x3 with modified RIPA buffer, and boiled in sample buffer for 5 min. Proteins were resolved by SDS-PAGE on an 8-16% Tris-Glycine gradient gel (Novex Inc., San Diego, CA) using 25μg protein/lane, and transferred (30v x 3h) to polyvinylidene fluoride membrane (PVDF). The blot was blocked (3% dry milk in PBS, 1h, RT) and probed with specific antibodies raised against established components of the zonula adherens including: α-, β-, and γ-catenin, $p120^{Cas}$, and cadherin. The blot was subsequently incubated with HRP-conjugated anti-mouse IgG (0.13μg/ml) (Transduction Laboratories). The blot was ten rinsed and developed with enhanced chemiluminescence (ECL) (Amersham Life Sciences, Arlington Heights, IL) and exposed to DuPont Reflection (NEF-406) film.

<u>Expression of Paxillin-GST Fusion Protein:</u> RNA was isolated from bovine pulmonary artery EC (ATCC) using RNAzol (Tel-Test, Inc., Friendswood, TX) and 1μg of the RNA was reverse transcribed into single stranded cDNA following the

manufacturer's recommendations (Gibco BRL Inc., Grand Island, NY). The full coding sequence corresponding to paxillin was cloned from this cDNA library. Amplification primers were targeted to sequences beginning 6 bases upstream from the start codon (TTTATCGAATTCCCGGCCATGGACGACCTCGAC) and ending 3 bases downstream from the stop codon of the paxillin cDNA (GTAACTGTCGACCACCTAGCAGAAGAGCTTGAGGAAGC); these primers introduced EcoR I (5') and Sal I (3') restriction sites (underlined) needed for subsequent sub-cloning. The PCR product was directly sub-cloned into the TA cloning vector (Invitrogen, San Diego, CA); excised by cleavage with EcoR I (5') and Sal I (3'); and sub-cloned into pGEX-5X-1 (Pharmacia Biotech, Inc., Piscataway, NJ) for production of a recombinant paxillin-GST fusion-protein in *Escherichia coli* (DH5α). Protein expression was induced for 3h by the addition of 0.25mM isopropyl-β-D-thiogalactopyranose. Cells were harvested by centrifugation at 10,000g for 12min. The bacteria were lysed with TBS containing 2mg/ml lysozyme, 0.1% β-mercaptoethanol, and protease inhibitors for 30min at room temperature. The buffer containing the lysates was brought up to a final concentration of 1% Triton X-100 and incubated on ice for 30min. The lysates were centrifuged at 10,000g for 15min. The fusion protein was recovered from the supernatant by incubation with glutathione-Sepharose 4B (Pharmacia).

GST-Paxillin Pull Down Assay: Lysates from LPS-exposed and media control EC were incubated with GST-paxillin immobilized on sepharose beads at 4°C,

overnight. The beads were removed, washed, and bound proteins eluted. The proteins were resolved by SDS-PAGE and transferred to PVDF. The blot was probed with anti-β catenin antibody followed by HRP-conjugated anti-mouse IgG and then developed by ECL.

Coimmunoprecipitation of Cadherin-Binding Poteins to Evaluate the State of Assembly of the Zonula Adherens: Lysates from EC exposed to varying concentrations of LPS for increasing exposure times and **simultaneous** media **controls were** immunoprecipitated with pancadherin antibody as described above. The cadherin immunoprecipitates and any cadherin-binding proteins were resolved by SDS-PAGE and transferred to PVDF. The blots were incubated with specific antibodies raised against α-, β-, and γ catenin and $p120^{Cas}$ followed by HRP-conjugated anti-mouse IgG and developed with ECL.

B. RESULTS:

Coimmunoprecipitation for Paxillin-Binding Proteins: Paxillin immunoprecipitates were harvested from lysates of LPS-exposed and media control EC and were probed for cadhein, α-, β-, γ-catenin and $p120^{Cas}$. For most of these ZA components tested, we could not clearly demonstrate that LPS treatment consistently and significantly increased binding to paxillin compared to the media controls under moderate detergent conditions. In preliminary experiments, paxillin immunoprecipitates probed for β-catenin revealed increased coimmunoprecipitation of β-catenin in lysates from LPS-exposed *vs* media control EC (Figure 1).

GST-Paxillin Pull Down Assay: Lysates from LPS-exposed EC contained no more ZA proteins that bound to GST-paxillin than did lysates from media control EC. One exception was that β-catenin from lysates from LPS-exposed EC bound more to full length GST-paxillin than did β-catenin from media control EC (Figure 2). No difference in binding could be demonstrated when a truncated GST-paxillin that only contained the first 3 tyrosines was used (up to residue 237).

Coimmunoprecipitation Cadherin-Binding Proteins: Cadherin immunoprecipitates were harvested from lysates of LPS-exposed and media control EC and were probed for α-, β-, γ-catenin and $p120^{Cas}$. These studies did not reveal evidence of increased ZA disassembly in LPS-treated EC. In these same studies, LPS exposure of $\geq$2h was associated with 2 immunoreactive β-catenin bands with MW of 95kDa and 75kDa (Figure 3) and 3 immunoreactive γ-catenin bands with MW of 82kDa, 72kDa, and 65kDa (Figure 4). Prior treatment with either of two structurally and functionally dissimilar protein tyrosine kinase inhibitors, herbimycin A (1μM) and genistein (50μg/ml), did not prevent the appearance of these additional bands (data not shown).

(7) CONCLUSIONS:

a. In preliminary experiments, LPS increases paxillin binding to β-catenin in EC by either coimmunoprecipitation or GST-paxillin pull down techniques.

b. LPS does not induce ZA disassembly in EC.

c. LPS does induce additional immunoreactive β- and γ-catenin bands. Whether these additional bands with increased gel mobility result from protease-mediated cleavage and/or alternately spliced products is not yet known. This LPS effect was not tyrosine phosphorylation-dependent.

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(9) APPENDIX:

Figures 1 - 4

Figure 1

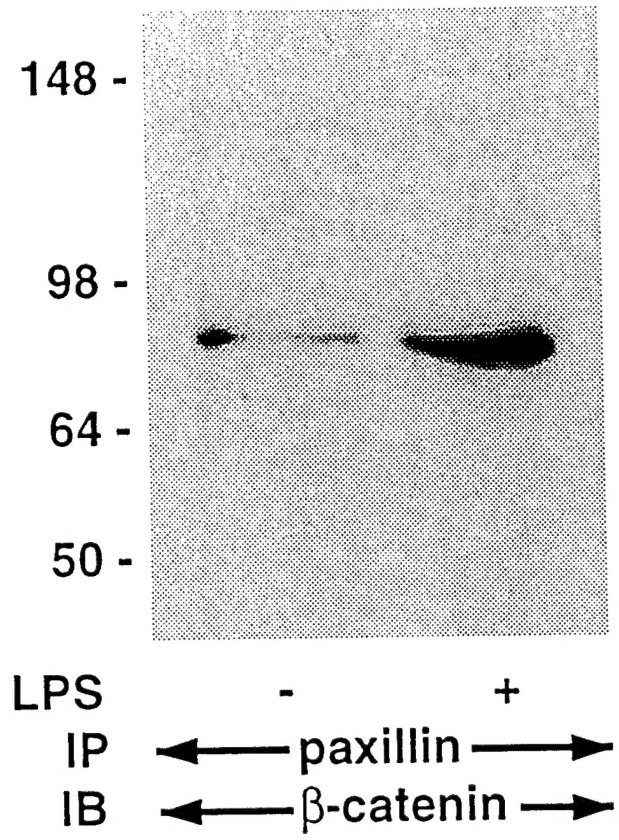
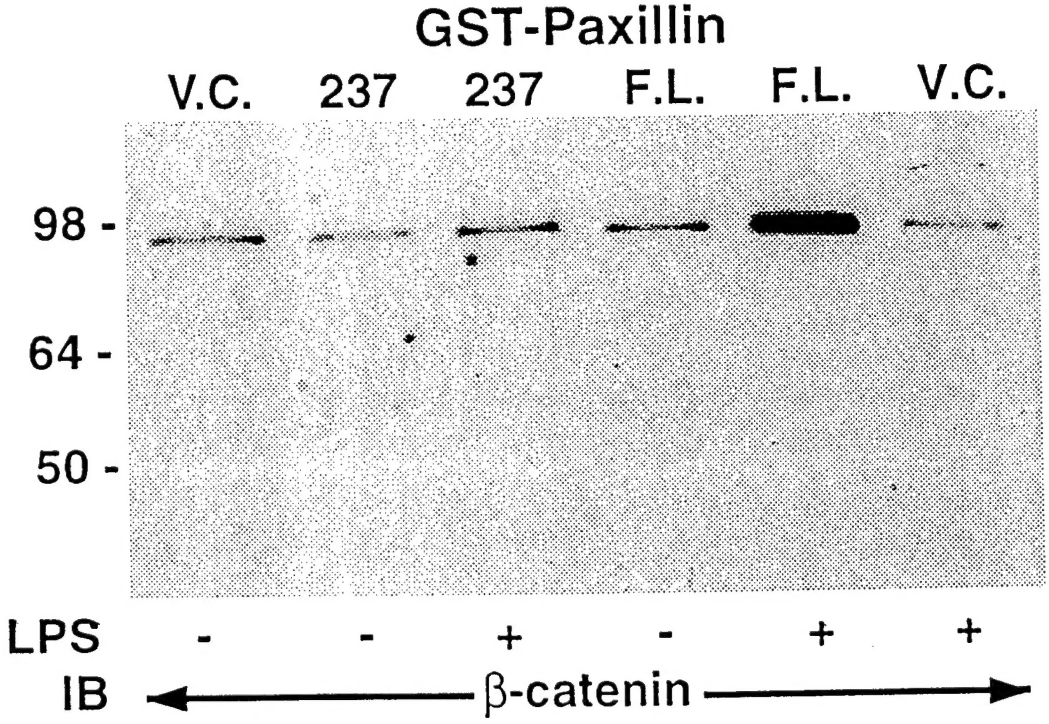


Figure 2



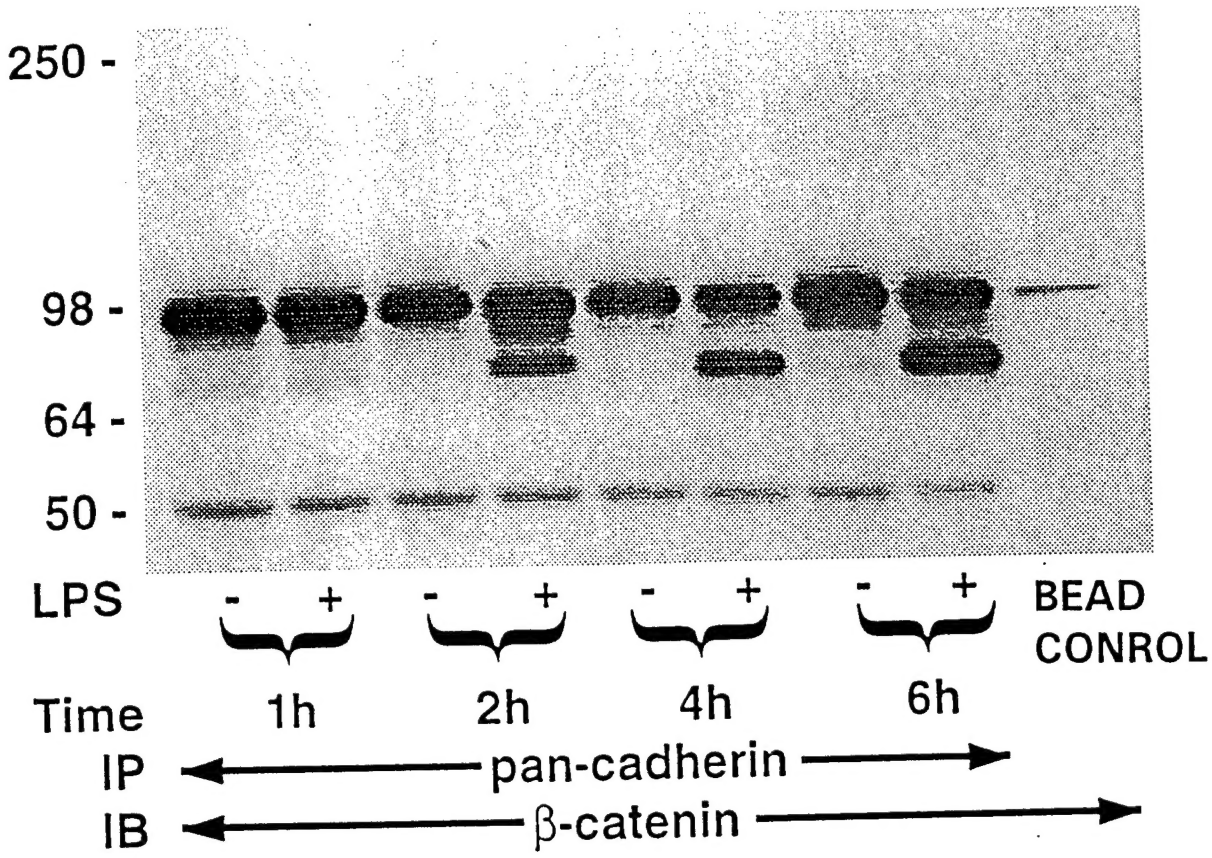


Figure 3

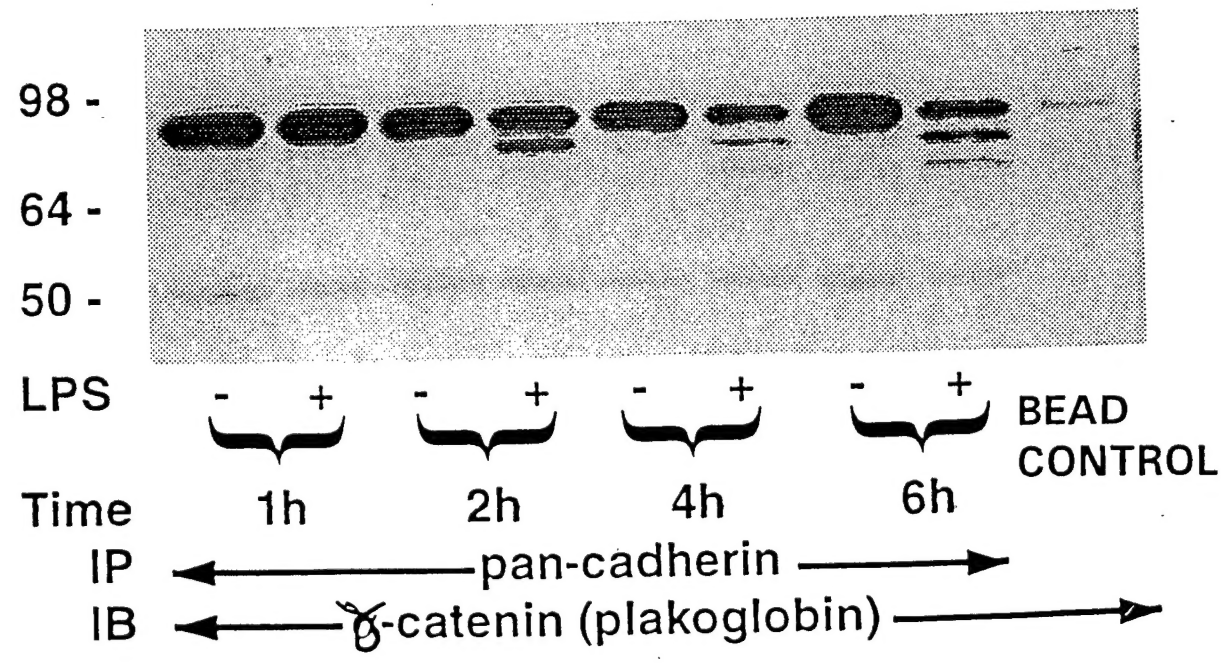


Figure 4